Journal of Medical Virology 57:259–263 (1999)

High Prevalence of TT Virus Infection in Brazilian Blood Donors

Christian Niel,[1]* Jaqueline M. de Oliveira,[1] Rudolf S. Ross,[2] Selma A. Gomes,[1] Michael Roggendorf,[2] and Sergei Viazov[2]

[1]Department of Virology, Oswaldo Cruz Institute, Rio de Janeiro, Brazil
[2]Institute of Virology, University of Essen, Germany

A recent report has described the molecular cloning and characterization of a novel, single-stranded DNA virus, named TT virus (TTV), which was present in the sera of Japanese patients with posttransfusion hepatitis of unknown etiology [Okamoto et al. (1998) Hepatology Research 10:1–16]. Using a nested polymerase chain reaction assay, sera from Brazilian patients with acute non A-C hepatitis and blood donors were examined for the presence of TTV DNA sequences. Thirty-seven of 52 (71%) patients with acute non A-C hepatitis and 45 of 72 (62%) blood donors were found to have TTV sequences in their sera. Such a high proportion in blood donors indicated that TTV infection is common in the general Brazilian population. Partial nucleotide sequences (326 bases in open reading frame 1) from seven isolates were determined. By phylogenetic analysis, four TTV strains were classified into the genomic subgroup G1a described previously. The three others belonged to subgroup G1b. Sequence homologies between strains belonging to a same subgroup were 92.9–99.1%, whereas homologies of 85.9–90.2% were calculated between isolates from different subgroups. ***J. Med. Virol. 57: 259–263, 1999.*** © 1999 Wiley-Liss, Inc.

KEY WORDS: TTV DNA; hepatitis; polymerase chain reaction; nucleotide sequencing; phylogenetic analysis; genotypes

INTRODUCTION

A number of viruses, including hepatitis A to E viruses, cytomegalovirus (CMV), herpesvirus, and Epstein-Barr virus, are known to cause hepatitis in humans. However, as some cases of posttransfusion hepatitis occur in the absence of serological markers for these viruses, the existence of other potentially causative viral agents has been suspected and investigated. Using representational difference analysis [Lisitsyn et al., 1993; Muerhoff et al., 1997], two novel viruses have been isolated recently from patients with hepatitis.

First, a flavivirus named GB virus C (GBV-C) and another isolate of GBV-C, hepatitis G virus (HGV), have been isolated from the sera of patients with non A-C hepatitis [Simons et al., 1995; Linnen et al., 1996]. However, further studies have shown that GBV-C/HGV does not account for a significant part of non A-E hepatitis cases [Alter H et al., 1997; Alter M et al., 1997].

On the other hand, a DNA has been cloned from the serum of a Japanese patient with posttransfusion hepatitis of unknown etiology [Nishizawa et al., 1997]. This DNA constituted the genome of a novel virus named TT virus (TTV) after the initials of the patient. TTV appeared to be a single-stranded DNA virus with a genome length of 3,739 bases. Two open reading frames, ORF1 and ORF2, capable of encoding 770 and 202 amino acids, respectively, have been identified [Okamoto et al., 1998]. Although some data have been obtained that would indicate resemblance of TTV to parvoviruses, no significant sequence homology between TTV genome and any reported sequences has been found, and TTV has not yet been classified. In three patients, the titers of TTV DNA correlated with the elevation of aminotransferase levels during the course of posttransfusion hepatitis. The possibility of TTV to induce non A to G hepatitis has thus been suggested [Nishizawa et al., 1997].

By comparison of partial TTV nucleotide sequences from Japanese isolates, the existence of two genomic groups, G1 and G2, has been demonstrated. Each of

The sequences reported in this paper have been deposited in the Genbank database under accession numbers AF081078 to AF081084.

Grant sponsors: the PROBRAL CAPES/DAAD collaboration program; CNPq; and FAPERJ.

*Correspondence to: Christian Niel, Department of Virology, Oswaldo Cruz Institute, FIOCRUZ, Avenida Brasil 4365, 21040-900 Rio de Janeiro, Brazil. E-mail: niel@gene.dbbm.fiocruz.br

Accepted 17 August 1998

these genomic groups was further divided into two subgroups differing by 11–15% in sequence [Okamoto et al., 1998].

The presence of TTV DNA was demonstrated in the sera of a majority of Brazilian patients with acute non A-C hepatitis and healthy blood donors. Genomic subgroups G1a and G1b were shown to circulate in Brazil.

MATERIALS AND METHODS

Population Studied

Of a total of about 6,500 patients referred to the Brazilian Reference Center for Viral Hepatitis (BRCVF) between 1989 and 1992, 1,617 patients had acute hepatitis. Fifty-two presented acute non A-C hepatitis defined by the absence of serological markers for hepatitis A virus (anti-HAV IgM), hepatitis B virus (HBsAg and anti-HBc IgM), HCV (anti-HCV), and CMV (anti-CMV IgM). Presence of TTV DNA was investigated in the sera of these patients. Seventy-two voluntary blood donors, whose blood was collected in 1997 in the city of Rio de Janeiro, were enrolled as a control group. Other patients with elevated levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), were randomly selected among the patients referred to the BRCVF.

DNA Extraction and Polymerase Chain Reaction

Total DNA was purified from 200 μl serum by use of the QIAamp blood kit (Qiagen, Hilden, Germany) following the manufacturer's instructions: Briefly, biological membranes and viruses were lysed by the addition of protease and incubation at 70°C for 10 min. After the addition of ethanol, DNA was bound to a silica membrane, washed two times, and finally eluted in 50 μl distilled water. Five microliters were added to a first round of polymerase chain reaction (PCR) performed for 35 cycles at 94°C, 1 min; 50°C, 1 min; 72°C, 2 min (and an additional 7 min at 72°C in the last cycle) in a final volume of 50 μl. One microliter of the PCR product was submitted to nested PCR. This reaction was carried out for 35 cycles under the same conditions (only increasing annealing temperature to 55°C). DNA extracted from each sample was amplified into two separate PCR experiments (A and B). In experiment A, the primers used were NG001–RD038 (external, first round) and RD037, RD051, and RD052 (internal, second round). These primers have been previously described [Nishizawa et al., 1997]. In experiment B, the primers used were derived from primers NG059, NG061, and NG063 [Okamoto et al., 1998]. These were degenerated as follows in order to amplify sequences from different TTV genotypes. External sense (first round): 5′ ACAGACAGRGGMGRAGGNAAYATG 3′, nt 1900 to 1923; Internal sense (second round): 5′ GGNAAYATGYTRTGGATAGACTGG 3′, nt 1915 to 1938; Antisense (both rounds): 5′ CTGGCATYTTWCCRTTTCCAAARTT 3′, nt 2185 to 2161. Seven microliters of amplification products were electrophoresed in a 1.8% agarose gel, stained with ethidium bromide and visualized under ultraviolet light.

Nucleotide Sequencing

One microliter of the amplification product from the first round of experiment A was submitted to a semi-nested PCR assay undertaken with primers NG001 and RD052 in a final volume of 100 μl. PCR conditions were the same as above, only decreasing annealing temperature to 45°C. The entire amplification product was run on a 1% agarose gel, and the unique DNA band of 396 base pairs (bp) was cut off the gel. DNA was purified using the QIAquickgel extraction kit (Qiagen) and directly sequenced from both directions using Prizm Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Perkin Elmer, Norwalk, CT).

Phylogenetic Analysis

Nucleotide sequences were aligned using PILEUP (Wisconsin Sequence Analysis Package, GCG, Madison, WI). This program uses the unweighted pair-group method with arithmetic averages (UPGMA) procedure for the construction of phylogenetic trees.

Statistical Analysis

The 95% confidence intervals of proportions were calculated using Epi Info 6.03 program (Centers for Disease Control, Atlanta, GA, and World Health Organization, Geneva, Switzerland).

RESULTS

The presence of TTV DNA was sought in the sera of patients with acute non A-C hepatitis. Since the first report on TTV [Nishizawa et al., 1997] mentioned the association between TTV infection and elevation of transaminase levels, individuals with elevated transaminases but without acute hepatitis, were also examined.

Table I shows the demographic, biochemical, and clinical data of 43 patients (19 male and 24 female, aged from 1 to 78 years) whose sera were found to contain TTV DNA. Only five patients had received blood transfusion. Thirty-seven patients had acute non A-C hepatitis, two presented chronic hepatitis, and four were asymptomatic. Two of the asymptomatic TTV infected patients (numbers 16315 and 16478) were blood donors with elevated transaminases. It was then decided to seek the presence of TTV DNA in sera from normal blood donors. Table II shows the prevalence of TTV infection in 72 healthy blood donors, taken as a control group, and comparison with a group of 52 consecutive patients with acute non A-C hepatitis. The results showed a surprisingly high (62%) prevalence of TTV-infected individuals among the "healthy" blood donors. The proportion of TTV-infected individuals in the group of acute non A-C hepatitis patients was also high (71%) and not significantly different. These figures were obtained with a set of degenerated primers (experiment B, see Materials and Methods). When another set of PCR primers was used (experiment A), the

TABLE I. Demographic, Biomedical, and Clinical Data of 43 TTV-Infected Patients

Patient number	Sex	Age (years)	ALT	AST	Blood transfusion	Onset of symptoms
Acute non A-C hepatitis						
4414	F	60	360	130	No	5 days
5153	M	51	150	100	No	3 months
5178	M	29	183	182	No	20 days
5201	M	78	98	92	No	26 days
5249	F	25	271	66	No	N.A.
5281	F	42	24	95	Yes	5 months
5342	M	9	120	90	No	9 days
5519	M	51	400	150	No	3 months
5665	F	62	205	86	No	10 days
5713	F	42	82	190	No	4 months
6044	F	30	330	80	No	5 days
6104	F	39	180	111	No	30 days
6182	M	1	380	328	No	4 months
6251	F	47	2350	1300	No	15 days
6558	M	20	126	53	No	N.A.
6718	F	62	1123	1019	Multiple	27 days
6800	F	33	115	64	No	7 days
6934	F	24	400	290	No	5 days
6949	F	52	93	170	No	30 days
6964	M	54	260	178	No	7 days
6967	M	52	153	124	No	N.A.
6970	M	27	75	29	No	4 months
			N.A.			
7009	F	29		N.A.	Yes	2 months
7139	F	49	130	105	No	30 days
7239	F	45	60	N.A.	Yes	2 months
7274	F	29	694	809	No	36 days
7419	F	48	252	196	No	14 days
7508	F	21	1230	1560	No	N.A.
7517	F	51	675	173	No	15 days
7519	M	45	440	132	No	16 days
7544	F	25	61	93	No	8 days
7761	F	33	723	390	No	15 days
8252	F	41	150	300	No	5 days
16250	M	18	1432	246	No	4 months
16272	M	53	192	186	No	21 days
16873	F	32	150	90	Yes	41 days
16952	M	43	57	60	No	2 months
Chronic hepatitis						
16207	M	47	215	108	No	3 years
16336	M	42	72	41	No	2 years
Asymptomatic						
						—
16290	M	68	87	120	No	
						—
16315	M	42	132	67	No	
						—
16320	F	55	96	64	No	
16478	M	57	47	67	No	—

ALT, alanine transaminase; AST, aspartate transaminase; N.A., Not available.

TABLE II. Prevalence of TTV DNA-Positive Individuals

Group	*n*	Prevalence (CI[a]) %
Blood donors	72	62 (50 to 74)
Acute non A-C hepatitis	52	71 (57 to 83)

[a]CI, 95% confidence interval.

rate of serum TTV DNA detection was much lower (prevalence of 15–17% in both groups).

PCR products resulting from experiment A and corresponding to a genome fragment of 326 bases in ORF1, were sequenced directly. The sequencing was carried out on seven TTV isolates: three from patients with acute non A-C hepatitis and four from normal blood donors. These sequences, aligned with those of two Japanese isolates [Nishizawa et al., 1997], appear in Figure 1. Most nucleotide changes occurred in the third position of codons. On a total of 142 mutations against consensus sequence, 27% were on the first position, 8% on the second, and 65% on the third. By phylogenetic analysis, Brazilian strains were classified into two genomic subgroups, with four strains in subgroup G1a and three isolates in G1b (Fig. 2). In each of these subgroups, the presence of TTV sequences derived from both normal blood donors and hepatitis pa-

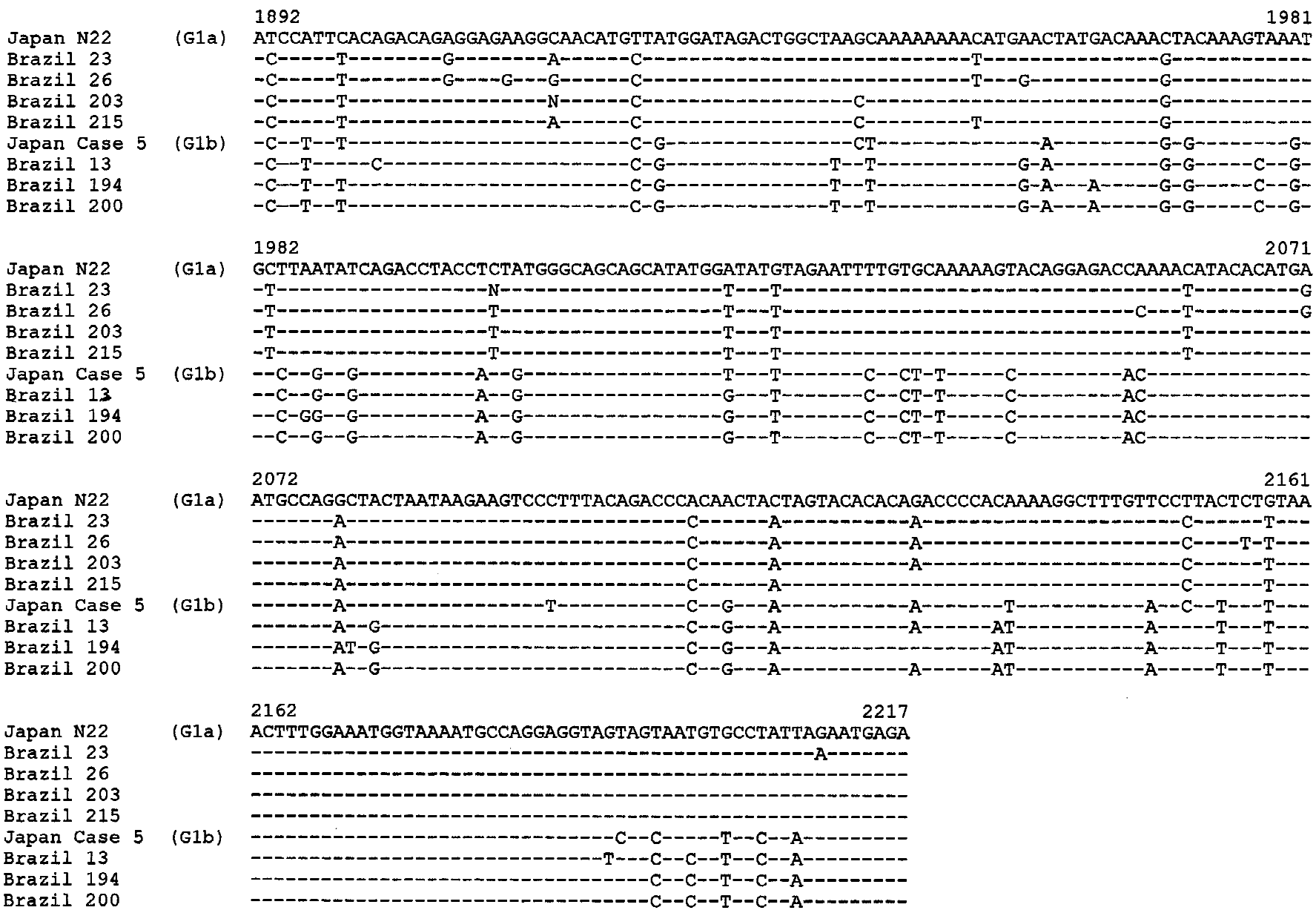

Fig. 1. Partial nucleotide sequences (nucleotides 1892–2217) of Brazilian TT virus isolates and comparison with the corresponding sequences of two Japanese isolates.

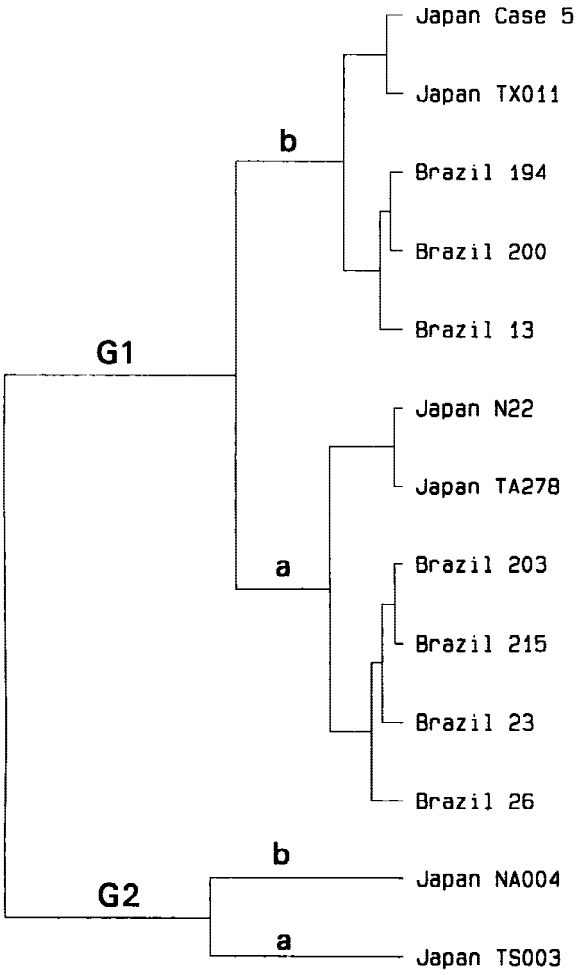

Fig. 2. Phylogenetic tree (UPGMA) of seven Brazilian and six Japanese TT virus isolates based on partial nucleotide sequences. G1a, G1b, G2a, and G2b are genomic subgroups [Okamoto et al., 1998]. Strains numbers 194, 200, 203, and 215 were from normal blood donors, the other Brazilian strains were from patients with liver disease.

tients could be noted. Sequence homologies between strains belonging to a same subgroup were from 92.9% to 99.1%, whereas homologies between isolates from subgroups G1a and G1b were from 85.9% to 90.2%. In each subgroup, sequence homologies were higher among isolates from the same country than between Brazilian and Japanese strains.

DISCUSSION

TTV was discovered recently by means of representational difference analysis [Nishizawa et al., 1997]. After picobirnaviruses [Pereira et al., 1988] and GBV-C/HGV [Simons et al., 1995; Linnen et al., 1996], TTV is another virus whose genome has been characterized before detection of viral particles and antigens. At this time, TTV has only been reported in the Far East. In Japan, its presence has been detected at a very high prevalence (39–68%) in the sera of patients with non A-G fulminant hepatitis and chronic liver disease, persons with hemophilia, intravenous drug users, and hemodialysis patients [Okamoto et al., 1998]. TTV infection has also been demonstrated in Japanese blood donors, although at a lower prevalence (12%).

TTV was also found among Brazilian blood donors and patients with acute non A-C hepatitis. As for GBV-C/HGV [Lampe et al., 1998], the prevalence of TTV infection in Brazilian blood donors was much higher (62%) than that reported for Japanese blood donors. Although the TTV prevalence was slightly higher in the group of patients with acute non A-C hepatitis than in normal blood donors, it is not possible, from our results, to establish a link between TTV infection and liver disease.

The high prevalence of TTV infection in blood donors was calculated from PCR experiments using a set of degenerated primers (experiment B). The TTV identity of such amplified DNA products was confirmed by nucleotide sequencing of some isolates (not shown).

TTV has been shown to be a transfusion-transmissible virus. Because a large majority of our TTV infected patients had not received blood transfusion (Table I), other routes of transmission must exist that remain to be determined.

TTV isolates from at least two genomic subgroups, G1a and G1b, are circulating in Brazil. These two subgroups are the most commonly found in Japan [Okamoto et al., 1998]. Sequencing of a larger number of TTV strains from South America would indicate if other subgroups are circulating on this continent.

REFERENCES

Alter HJ, Nakatsuji Y, Melpolder J, Wages J, Wesley R, Shih W-K, Kim JP. 1997. The incidence of transfusion-associated hepatitis G virus infection and its relation to liver disease. N Engl J Med 336:747–754.

Alter MJ, Gallagher M, Morris TT, Moyer LA, Meeks EL, Krawczynski K, Kim JP, Margolis H. 1997. Acute non A-E hepatitis in the United States and the role of hepatitis G virus infection. N Engl J Med 336:741–746.

Lampe E, de Oliveira JM, Pereira JL, Saback FL, Yoshida CFT, Niel C. 1998. Hepatitis G virus (GBV-C) infection among Brazilian patients with chronic liver disease and blood donors. Clin Diagn Virol 9:1–7.

Linnen J, Wages J Jr, Zhang-Keck Z-Y, Fry KE, Krawczynski KZ, Alter H, Koonin E, Gallagher M, Alter M, Hadziyannis S, Karayiannis P, Fung K, Nakatsuji Y, Shih JW-K, Young L, Piatak M Jr, Hoover C, Fernandez J, Chen S, Zou J-C, Morris T, Hyams KC, Ismay S, Lifson JD, Hess G, Foung SKH, Thomas H, Bradley D, Margolis H, Kim JP. 1996. Molecular cloning and disease association of hepatitis G virus: a transfusion-transmissible agent. Science 271:505–508.

Lisitsyn N, Lisitsyn N, Wigler M. 1993. Cloning the differences between two complex genomes. Science 259:946–951.

Muerhoff AS, Leary TP, Desai SM, Mushahwar IK. 1997. Amplification and subtraction methods and their application to the discovery of novel human viruses. J Med Virol 53:96–103.

Nishizawa T, Okamoto H, Konishi K, Yoshizawa H, Miyakawa Y, Mayumi M. 1997. A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. Biochem Biophys Res Commun 241:92–97.

Okamoto H, Nishizawa T, Kato N, Ukita M, Ikeda H, Iizuka H, Miyakawa Y, Mayumi M. 1998. Molecular cloning and characterization of a novel DNA virus (TTV) associated with posttransfusion hepatitis of unknown etiology. Hepatol Res 10:1–16.

Pereira HG, Fialho AM, Flewett TH, Teixeira JM, Andrade ZP. 1988. Novel viruses in human faeces. Lancet 2:103–104.

Simons JN, Leary TP, Dawson GJ, Pilot-Matias TJ, Muerhoff AS, Schlauder GG, Desai SM, Mushahwar IK. 1995. Isolation of novel virus-like sequences associated with human hepatitis. Nat Med 1:564–569.